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MICROBIAL REDUCTIVE DECHLORINATION OF HEXACHLORO-1,3-BUTADIENE IN A METHANOGENIC ENRICHMENT CULTURE

RANDALL S. BOOKER[M] and SPYROS G. PAVLOSTATHIS*[M]
School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA 30332-0512, USA

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Abstract—Sequential reductive dechlorination of hexachloro-1,3-butadiene (HCBD) was achieved by a mixed, methanogenic culture enriched from a contaminated estuarine sediment. Both methanol and lactate served as carbon and electron sources. Methanol was stoichiometrically converted to methane, whereas lactate was fermented to propionate and acetate and then to methane. Lactate and propionate fermentation, as well as methanogenesis were not inhibited at 0.4 mg HCBD/l, the normal enrichment culture HCBD feeding level. At a higher initial HCBD level of 1.5 mg HCBD/l, propionate fermentation and acetoclastic methanogenesis were inhibited while, after a lag time, enhanced HCBD dechlorination rates were observed. While lactate fermentation was not inhibited at high concentrations (> 25 mM) of 2-bromoethanesulfonate (BES), both propionate fermentation and methanogenesis were completely inhibited, although the HCBD dechlorination rate was not affected. Therefore, methanogens were not likely responsible for the observed dechlorination of HCBD in the enrichment culture. The predominant HCBD dechlorination products were isomers of tri- and dichloro-1,3-butadiene. Traces of a monochloro-1,3-butadiene isomer were also detected. Although extensive dechlorination of HCBD was achieved by the enrichment culture, the detoxification efficiency of this process remains unclear because the potential inhibitory effect of the HCBD transformation products is unknown.

Key words—fermentation, hexachloro-1,3-butadiene, inhibition, methanogenesis, reductive dechlorination

INTRODUCTION

Hexachloro-1,3-butadiene (HCBD) is a priority pollutant found in waters, soils, sediments and aquatic organisms worldwide (Laseter *et al.*, 1976; Oliver and Charlton, 1984; Pereira *et al.*, 1988; Murray and Beck, 1989; Botta *et al.*, 1996; Prytula and Pavlostathis, 1996; Gess and Pavlostathis, 1997). In the USA, HCBD was originally used in the rubber industry, where it served as a chlorine recovery solvent in the production and processing of synthetic and natural rubber. HCBD has also been used as a heat transfer and transformer fluid, hydraulic fluid, and solvent for the removal of hydrocarbons, as well as an insecticide, herbicide, fungicide, and algicide (Anonymous, 1992; Verschueren, 1996). Although HCBD has not been intentionally manufactured in the USA since 1970 (Anonymous, 1992), it is currently generated in environmentally significant quantities as a by-product during the production of volatile chlorinated solvents such as tetra- and trichloroethene and carbon tetrachloride.

The structure of HCBD is presented in Fig. 1. HCBD is hydrophobic with low water solubility (2 mg/l at 20°C) and high volatility (Henry's Law constant = 2.57×10^{-2} atm m^3/mol at 20°C) (Mackay *et al.*, 1993). Because of its relatively high octanol-water partition coefficient (log $K_{ow} = 4.78$) (Mackay *et al.*, 1993), HCBD tends to sorb and accumulate in sediments and the lipid tissues of aquatic organisms (Pereira *et al.*, 1988). Consequently, contaminated sediments act as a source of long-term chronic contamination leading to the bioaccumulation of HCBD in aquatic organisms (Pereira *et al.*, 1988; Murray and Beck, 1989). HCBD is toxic when inhaled, ingested or absorbed through the skin, and is considered carcinogenic, neurotoxic, fetotoxic, and mutagenic, as well as the most nephrotoxic aliphatic chlorinated hydrocarbon (Anonymous, 1992).

In spite of the extensive presence of HCBD as a contaminant worldwide and its well-known deleterious health effects, very little information concerning the fate of HCBD in either aerobic or anaerobic

*Author to whom all correspondence should be addressed. Tel.: +1-404-894-9367; fax: +1-404-894-8266; e-mail: spyros.pavlostathis@ce.gatech.edu

environments has been reported. To date, the only reliable evidence of microbially mediated reductive dechlorination of HCBD has been reported by Bosma *et al.* (1994) in a methanogenic sediment column. The lack of information on the biodegradation potential and environmental consequences of HCBD warrants further investigation. In particular, the extent of HCBD dechlorination, and the impact of the mobility and the potential toxicity of its biotransformation products on both natural and engineered systems may have significant ramifications.

The objectives of the work reported here were to: (a) assess the extent of the sequential reductive dechlorination of HCBD in a mixed, methanogenic enrichment culture; and (b) assess the effects of increasing concentrations of HCBD and 2-bromoethanesulfonate (BES—a selective inhibitor of methanogenesis), as well as the types of electron donor used on the kinetics of both HCBD dechlorination and exogenous substrate utilization.

MATERIALS AND METHODS

Chemicals

HCBD (97%) and 2-bromoethanesulfonate (BES) sodium salt were obtained from Aldrich Chemical (Milwaukee, WI, USA). HCBD stock solutions were prepared by dissolving neat HCBD in HPLC-grade methanol (99.9%) obtained from Fisher Scientific (Pittsburgh, PA, USA). In addition to methanol, lactate (60% w/w sodium syrup; Fisher Scientific) was used as electron donor. 2-chloro-1,3-butadiene, commercially known as chloroprene (44% in xylene; Chem Service, West Chester, PA, USA) was used as a biotransformation product standard. Sodium azide (Fisher Scientific) was used to prepare biologically inactive controls.

Development of an HCBD-biotransforming culture

Sediment contaminated with HCBD and chlorinated benzenes was collected from an industrial canal flowing into Bayou d'Inde, a tributary of the Calcasieu River near Lake Charles, Louisiana, USA. A chemical plant discharged wastewater generated in the production of chlorinated aliphatic compounds into this canal. Location of sediment, as well as details on the sediment sampling and storage procedures have been reported elsewhere (Prytula and Pavlostathis, 1996; Gess and Pavlostathis, 1997).

The contaminated sediment served as inoculum to develop an anaerobic culture in a 9-l glass reactor (120 g dry weight sediment in 6-l media). The medium had the following composition (in mg/l): K_2HPO_4, 900; KH_2PO_4, 500; NH_4Cl, 500; $CaCl_2{\cdot}2H_2O$, 100; $MgCl_2{\cdot}6H_2O$, 200; $FeCl_2{\cdot}4H_2O$, 100;$Na_2S{\cdot}9H_2O$, 500; $NaHCO_3$, 1200; resazurin (a redox indicator), 2. Also, 1 ml/l each of vitamin (Wolin *et al.*, 1963) and trace metal (Mah and Smith, 1981) stock solutions were added. The culture was maintained semi-continuously (i.e., a volume of culture was periodically removed under completely mixed conditions and replaced with the same volume of reduced medium, along with additions of HCBD and electron donor) with an average hydraulic (and solids) retention time of 30 days. At the beginning of each six-day feeding cycle, lactate, yeast extract, and HCBD in methanol were added resulting in initial concentrations of 500, 25, 0.4, and 200 mg/l, respectively. The culture was kept in the dark at 22°C in a constant temperature room, and mixed once daily with a magnetic stirrer. After four months of operation, the culture was sediment-free and used in batch assays.

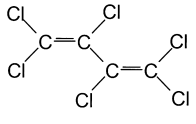

Fig. 1. Structure of hexachloro-1,3-butadiene (HCBD).

HCBD biotransformation assays

The biotransformation of HCBD by the enrichment culture was investigated in three separate assays which examined the effects of HCBD and BES concentrations as well as the type of exogenous substrate (methanol versus methanol + lactate), on the dechlorination of HCBD, gas production and organic acid fermentation. Because of the high volatility of HCBD, these assays were conducted in replicate 26-ml glass serum tubes which were periodically sacrificed for analyses. Aliquots of the enrichment culture were transferred to sealed, helium pre-flushed tubes using a helium-flushed syringe. Each tube was amended with an HCBD/methanol stock solution using a microsyringe. After all additions to each tube were made, the septum was replaced with another Teflon-lined septum under a helium atmosphere and the tube sealed with an aluminum crimp. All tubes were incubated at 22°C, and manually agitated once daily. Two HCBD control series—media only and azide-amended culture—were also used. The initial sodium azide concentration was 1 g/l.

In the first assay, in order to examine the role of methanol and lactate on HCBD dechlorination, two series were prepared with the same initial biomass and HCBD concentrations of 180 mg volatile suspended solids (VSS)/l and 0.4 mg/l, respectively. One series received only methanol (200 mg/l) whereas the other series received both methanol and lactate (200 and 500 mg/l, respectively). In a second assay, three series were prepared with initial HCBD levels of 0, 0.4 (normal enrichment culture feeding level), and 1.5 mg/l. All HCBD levels in this assay were below the aqueous solubility of HCBD. The HCBD levels represent nominal, initial HCBD levels which ignore liquid phase/headspace partitioning. For the purpose of simplicity and to avoid confusion, the term initial HCBD level, instead of concentration, is used throughout this work. Note that at every sampling time, culture tubes were sacrificed by injection of isooctane through the septum and the remaining HCBD and its transformation products were extracted and recovered (see below). Two HCBD stock solutions were prepared and all HCBD additions were made with the same volume of methanol (5 μl), thus providing an identical initial electron donor concentration in all three series and avoiding any possible inhibitory effects from increased methanol concentrations. In order to explore the role of methanogens in the biotransformation of HCBD, a third assay was conducted using initial BES concentrations of 0, 5, 25, and 50 mM. The four series were prepared with the same initial biomass (as VSS), HCBD, methanol and lactate concentrations (180, 0.4, 200 and 500 mg/l, respectively).

Analyses

HCBD and biotransformation products were recovered for analysis by solvent extraction. At each sampling time, individual culture tubes were extracted by injection of 2 ml isooctane through the septa followed by vigorous mixing. The tubes were then centrifuged at 3000 rpm for 15 min. The solvent layer was transferred to glass autosampler vials, and sealed with Teflon-lined septa and aluminum

crimps. Routine analysis of HCBD and less chlorinated biotransformation products was performed using an HP 5890 Series II gas chromatograph (GC) (Hewlett Packard, Palo Alto, CA, USA) equipped with an electron capture detector (ECD) and a 60-m DB-624 0.53-mm I.D. column (J&W Scientific, Folsom, CA, USA). The following temperature program was used: 100°C for 4 min, increase by 2°C/min to 210°C and hold for 6 min. Nitrogen was used as the carrier gas at a flow rate of 10 ml/min. 1,3,5-Tribromobenzene (TBB) was included in the isooctane and served as the internal standard. HCBD standard stock solutions in the range of 0.6 to 40,000 μg/l in methanol were used to develop a calibration curve which was then used to quantify HCBD in culture solvent extracts. The HCBD method detection limit was 0.3 μg/l. The efficiency of the solvent extraction procedure used in these assays was previously determined to be greater than 90% using azide-amended control samples.

Because chlorinated 1,3-butadiene standards, other than HCBD and 2-chloro-1,3-butadiene, are not commercially available, gas chromatography with mass spectrometric detection (GC–MS) was used to identify HCBD biotransformation products according to the number of chlorine substituents present. A Varian Star 3600cx GC with a Saturn 2000 Ion Trap Mass Spectrometer detector (Varian Analytical Instruments, Fernando, CA, USA) and a 30-m long, 0.25-mm I.D. DB-5 column was employed. Helium was the carrier gas at a flow rate of 1 ml/min and the following temperature program was used: 40°C for 5 min, increase by 1°C/min to 100°C. In order to achieve concentrations of HCBD transformation intermediates detectable by GC–MS, a 2-l culture was fed five-fold the normal HCBD concentration four times over a five-week period. Gas production was vented through a Tenax-TA (Supelco, Bellefonte, PA, USA) trap to recover volatile products. At the end of this experiment, the entire reactor was extracted with isooctane and the solvent phase recovered using a separatory funnel. The Tenax trap was extracted using hexane to desorb trapped, volatile HCBD transformation products. The culture and Tenax extracts were used in the GC–MS analysis of biotransformation products.

Gas production volume was measured by connecting the tube headspace via a needle to a water-filled buret and recording the volume of displaced water, after correcting to atmospheric pressure. Methane was determined by gas chromatography (thermal conductivity detector) as previously reported (Prytula and Pavlostathis, 1996). For the analysis of methanol, culture samples were syringe-filtered (0.2 μm) and analyzed using an HP 5890 Series II GC equipped with a flame ionization detector (FID) and a 10-m PoraPlot Q 0.53-mm I.D. column (Chrompack, Raritan, NJ, USA) operated isothermally at 70°C with nitrogen as the carrier gas. The method detection limit for methanol was 1 mg/l. Lactate, propionate, and acetate were analyzed using an HP Series 1050 high performance liquid chromatography (HPLC) unit equipped with a variable wavelength detector set for UV detection at 210 nm and an HPX-87H resin-based column (Bio-Rad, Richmond, CA, USA) operated isothermally at 65°C. Culture samples were acidified with an equal volume of 2.5% (v/v) phosphoric acid and filtered through a 0.2 μm polycarbonate membrane before HPLC analysis. Calibration curves for lactate, acetate and propionate were developed using standard stock solutions and the method detection limits were 9, 6, and 7 mg/l, respectively.

Particulate organic carbon (POC) was determined using a Shimadzu Total Organic Carbon (TOC) Analyzer equipped with a Solids Sample Module (SSM) and an infrared detector for CO_2 measurement (Shimadzu Instrument, Kyoto, Japan). Culture samples were filtered onto glass fiber filters, rinsed with deionized water, heated at 95°C for 10 min, and then combusted at 900°C. A correlation between culture POC and VSS was developed and used throughout this study to report biomass concentrations in terms of VSS. Oxidation-reduction potential (ORP), pH, chemical oxygen demand (COD), and VSS were measured following procedures outlined in Standard Methods (American Public Health Association, 1995). An Orion Research digital pH/millivolt meter model 611 was used in conjunction with a platinum electrode with an Ag/AgCl reference in 3.5 M KCl gel (Sensorex, Stanton, CA, USA) for ORP measurements.

RESULTS

Enrichment culture

The sequential biotransformation of HCBD was confirmed by the disappearance of the HCBD peak and appearance of a number of new peaks with shorter GC retention times (Fig. 2). Several peaks were identified as lesser chlorinated 1,3-butadienes using GC–MS, as discussed below. The microbial basis for the observed HCBD biotransformation was confirmed by using both abiotic (media only) and biotic (azide-amended cultures) controls.

Over the course of each feeding cycle of the enrichment culture, lactate and methanol were completely consumed within one day. Propionate and acetate were produced as a result of lactate fermen-

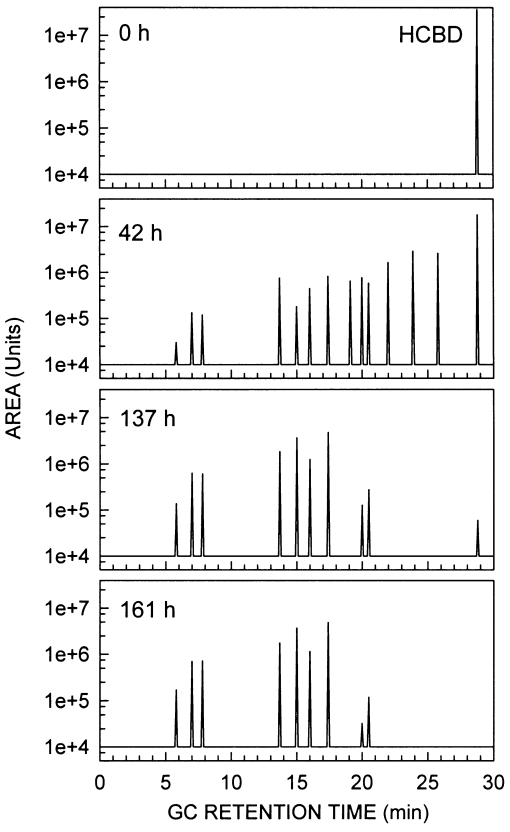

Fig. 2. Reproduced GC–ECD chromatograms showing the formation of HCBD dechlorination intermediates as a function of culture incubation time (complete solvent extraction of replicate sacrificial culture tubes).

tation and then propionate was converted to acetate. Both acetate and methanol were converted to methane. The methane content of the gas varied between 60 and 70%. The steady-state pH, ORP and biomass concentration values were 7.2 ± 0.2, -360 ± 20 mV and 247 ± 20 mg VSS/l, respectively (mean $\pm$ standard deviation; $n = 5$). By measuring initial and final values of both total and soluble COD during a feeding cycle, the observed microbial yield coefficient was determined as 0.12 mg biomass COD/mg soluble COD utilized. HCBD transformation activity in this culture was maintained for over three years.

HCBD biotransformation products

Fifteen transformation products were observed via GC–MS, including the following chlorinated homologs of 1,3-butadiene: one pentachloro-isomer, two tetrachloro-isomers, six trichloro-isomers, and six dichloro-isomers. Including the parent compound (i.e., HCBD), 14 major peaks were routinely identified in GC–ECD chromatograms (peak number; numbers in parenthesis denote increasing GC retention time in min $\pm$ 0.2 min): 1 (5.8), 2 (7.0), 3 (7.8), 4 (13.7), 5 (15.0), 6 (16.0), 7 (17.5), 8 (19.1), 9 (20.0), 10 (20.5), 11 (22.0), 12 (23.9), 13 (25.9), and 14 (28.8) (Fig. 2). Peak No. 14 is HCBD. Based on the match between the GC retention times of the observed peaks in both the GC–ECD and GC–MS chromatograms, peaks 1–3 were dichlorinated isomers, peaks 4–8 were trichlorinated isomers, and peaks 9–13 were tetra- and pentachlorinated isomers. Peak No. 13 was identified as one of the pentachlorinated isomers. Assuming similar GC–MS response factors for all chlorinated 1,3-butadiene homologs, isomers of tri- and dichloro-1,3-butadiene were observed as the predominant products at an incubation period of 6.7 days (Fig. 2). Trace amounts of 2-chloro-1,3-butadiene were also identified using the available standard and GC–ECD. Figure 3 shows the mass spectra of two chlorinated 1,3-butadiene isomers detected in solvent extracts of the HCBD dechlorinating enrichment culture.

Methanol and lactate as electron donors

The initial/mean final pH, ORP and biomass concentration values for this assay were 7.2/7.3, −300/−270 mV and 192/240 mg VSS/l, respectively. Both methanol and lactate were consumed within 2.4 days (data not shown). The fermentation pattern observed in previous assays (i.e., simultaneous production of propionate and acetate followed by conversion of propionate to acetate) was observed (data not shown). Acetate or other organic acids were not detected in the series amended with methanol only. Methane production is shown in Fig. 4(A). The methane content of the total gas produced was 67 and 62% for the methanol and methanol plus lactate amended series, respectively. A carbon balance equal to 1.05 (i.e., sum of methane-, carbon dioxide- and biomass-C produced divided by initial methanol-C) for the methanol-only series indicates that methanol was used mainly for methanogenesis and biosynthesis and secondarily for HCBD dechlorination.

The rate of HCBD biotransformation was not

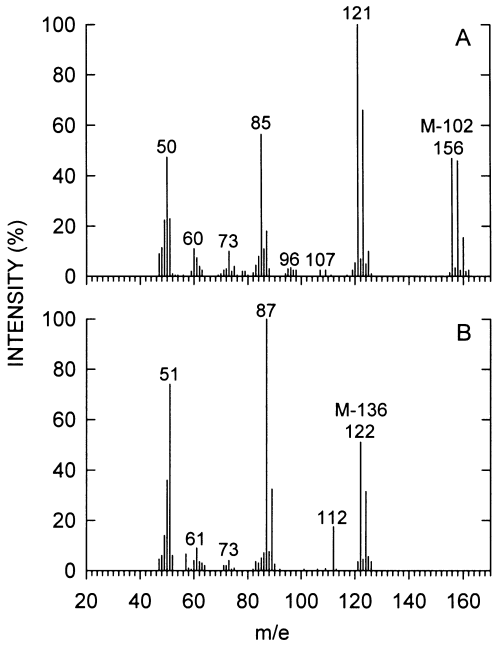

Fig. 3. Mass spectra of isomers of tri- (A) and dichloro-1,3-butadiene (B) detected in solvent extracts of the HCBD dechlorinating enrichment culture.

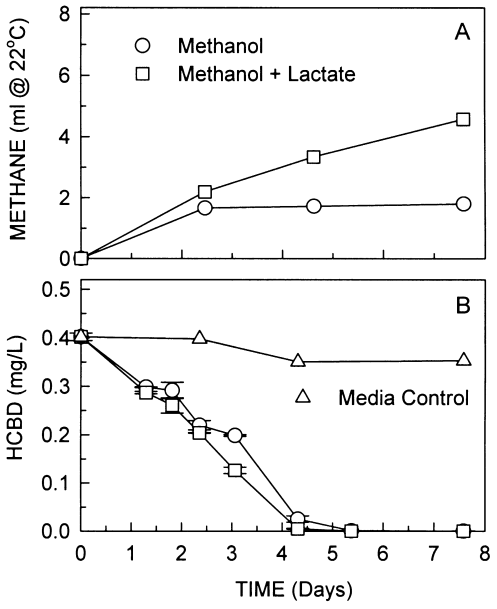

Fig. 4. Comparison of the effect of methanol vs methanol plus lactate on the methane production (A) and the HCBD biotransformation rate (B) (bars represent one standard deviation of the means).

Table 1. Total gas and methane production as well as biomass concentration at six days of incubation as a function of initial HCBD level

Initial HCBD level (mg/l)	Final biomass[a] (mg VSS/l)	Total gas production[b] (%)	Methane production[b] (%)	Methane content[c] (%)
0	269	100.0	100.0	60.0
0.4	267	99.5	99.2	60.2
1.5	258	67.7	71.8	56.6

[a]The mean initial biomass concentration in all three culture series was 219 mg VSS/l.
[b]Expressed as a percentage of the gas produced by the HCBD unamended culture series.
[c]Expressed as a percentage of the total gas produced by each culture series.

significantly different in the methanol and methanol plus lactate series (Fig. 4(B)). The net, biomass-normalized HCBD biotransformation rates were 0.40 ± 0.10 and 0.37 ± 0.04 μg HCBD/mg VSS-d, for the methanol and methanol plus lactate series, respectively ($\pm$95% confidence interval). These rates are not statistically different ($\alpha = 0.05$). The methanol-only series, provided with approximately 300 mg COD/l (=37.5 millielectron equivalents/l) in the form of methanol, yielded essentially the same biomass-normalized HCBD biotransformation rate as the 1000 mg lactate/l series, provided with 1367 mg COD/l (=171 millielectron equivalents/l) in the form of both methanol and lactate. Therefore, both culture series were kinetically saturated with respect to the concentration of available electrons for the biotransformation of HCBD—i.e., zero-order HCBD transformation kinetics with respect to the available reducing equivalents.

Effect of HCBD level

The initial/mean final pH and ORP values for this assay were 7.4/7.2 and −280/−260 mV, respectively. The effect of the initial HCBD on total gas and methane production, gas methane content, as well as biomass concentration at the end of the six-day incubation period, is shown in Table 1. All parameter values were similar for the zero and 0.4 mg HCBD/l series. However, when the initial HCBD level was increased to 1.5 mg/l, production of gas and its methane content declined (Table 1).

Figure 5 shows lactate and its fermentation products over the incubation period. Lactate was depleted in all three series within approximately 1.5 days, indicating that lactate fermentation was not inhibited by increasing initial HCBD levels. In the zero and 0.4 mg HCBD/l series, subsequent utilization of propionate and acetate was virtually identical. In these series, propionate fermentation was the limiting metabolic step, i.e., acetate was consumed faster than produced from propionate fermentation. At 1.5 mg HCBD/l, both propionate fermentation and acetoglastic methanogenesis appeared to be inhibited as consumption of propionate slowed and acetate accumulated over time (Fig. 5).

The effect of an increasing initial HCBD level on the rate of HCBD biotransformation is shown in Fig. 6. Over the ca 12-day incubation period, the HCBD concentration decline in both the media control and the azide-amended culture series was very low and similar, which indicates that the HCBD depletion in the biotic series was due to biotransformation. In addition, HCBD transformation products were not detected in solvent extracts of the two control series. Culture tubes amended with 0.4 mg HCBD/l—the normal HCBD feeding level of the enrichment culture—exhibited a rapid decrease in HCBD, with virtually complete HCBD removal occurring in approximately 3 days. The culture series with 1.5 mg HCBD/l initial HCBD level exhibited a different HCBD depletion pattern, characterized by an initial rapid decrease in HCBD, followed by a period with minimal biotransformation, which was followed by a period with a high transformation rate until all HCBD was depleted. Sequential dechlorination of HCBD took place in both the 0.4 and 1.5 mg HCBD/l series as confirmed by the observed peaks in GC–ECD chromatograms. Based on the previously discussed match

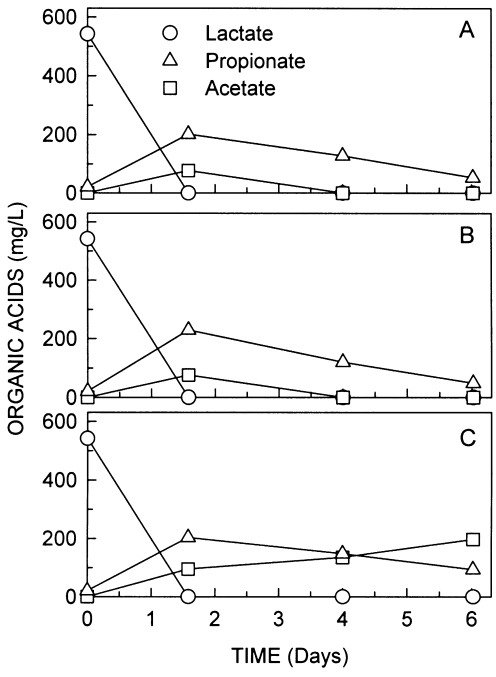

Fig. 5. Effect of initial HCBD level on lactate utilization, and the production and further utilization of propionate and acetate in three culture series amended with 0, 0.4 and 1.5 mg/l HCBD (A, B, and C, respectively).

between the peak retention times in both GC–MS and GC–ECD chromatograms, for any portion of HCBD transformed in these biotic series, the predominant transformation products at the end of the incubation period were isomers of tri- and dichloro-1,3-butadiene.

Net volumetric HCBD biotransformation rates normalized to the mean biomass concentration over the incubation period were 0.47 ± 0.09 and 1.44 ± 0.09 μg HCBD/mg VSS-d ($\pm$95% confidence interval) for the 0.4 and 1.5 mg HCBD/l series, respectively. Note that, for the 1.5 mg HCBD/l series, only data corresponding to the maximum sustained HCBD biotransformation rate were used. Therefore, compared to the normal HCBD feeding level of 0.4 mg/l, a higher HCBD biotransformation rate was achieved at the 1.5 mg HCBD/l initial level.

Effect of BES on HCBD dechlorination

The final pH values in the biotic series of this assay varied between 7.2 and 7.3. The effect of BES on methane production is shown in Fig. 7(A). The methane-to-total gas ratio was approximately 0.56 in the series with zero BES and decreased to 0.24, 0.18 and 0 with increasing BES concentrations to 5, 25, and 50 mM, respectively. Therefore at 50 mM BES, methanogenesis from both acetate and methanol was completely inhibited. Lactate was depleted in all four series within the first two days of incubation (data not shown). Propionate and acetate were not detected in the control (i.e., BES unamended series) at the end of the seven-day incubation period (data not shown). However, in the three BES-amended series, propionate and acetate accumulated (ranging from 193 to 224 and from 191 to 227 mg/l, respectively) and were not further utilized. The organic acids production and accumulation pattern in all three BES-amended series resembled that of the high HCBD level series (see Fig. 5(C)).

Despite the observed inhibition of propionate fermentation and methanogenesis in the BES-amended series, a significant difference in the HCBD biotransformation rate between the BES-amended and unamended series was not observed (Fig. 7(B)). Even at 50 mM BES, which resulted in complete inhibition of methanogenesis, the rate of HCBD biotransformation was not affected. It is noteworthy that no discernible difference between the BES-amended and the control culture series was observed in terms of the relative distribution of the HCBD biotransformation products at the end of the incubation period (i.e., the main products in all culture series were predominantly tri- and dichlorinated isomers). Therefore, addition of BES did not affect the dechlorination of either HCBD or its transformation products.

DISCUSSION

Sustained sequential reductive dechlorination of HCBD was achieved by the sediment-derived and enriched methanogenic consortium, resulting in production of primarily tri- and dichloro-1,3-butadiene isomers. To the best of our knowledge, no prior evidence of microbial reductive dechlorination of HCBD to predominantly trichloro- and dichloro-isomers, as well as traces of a monochlorinated isomer, has been reported in the literature. Bosma *et al.* (1994) observed that more than 90% of HCBD added to a sediment column operated under methanogenic conditions was transformed to tetrachloro-

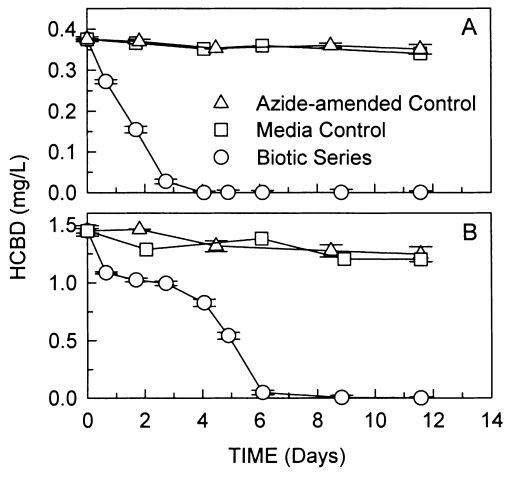

Fig. 6. Effect of initial HCBD level (A, 0.4 and B, 1.5 mg/l) on the biotransformation rate of HCBD (bars represent one standard deviation of the means).

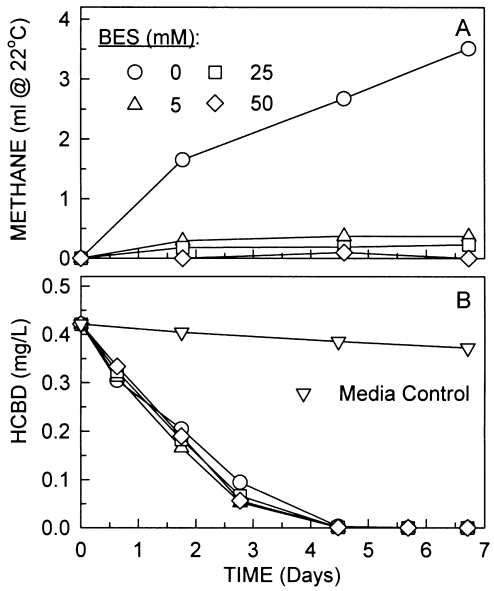

Fig. 7. Effect of increased BES concentrations on methane production (A) and HCBD biotransformation rate (B).

1,3-butadiene, and less than 5% was accounted for as trichloro-1,3-butadiene.

Lactate and methanol served as the primary electron donors for the HCBD-dechlorinating enrichment culture. Methanol was stoichiometrically converted to methane. As a result of lactate fermentation, both propionate and acetate were produced, and propionate was subsequently fermented to acetate. For a series of experiments, the carbon balances between the organic substrates used and products measured were above 94%. In addition, for short incubation periods, the molar ratio of propionate to acetate was 1.92 ± 0.35 (mean $\pm$ standard deviation; $n = 4$), which is very close to the theoretical ratio of two according to the pathway for the simultaneous fermentation of lactate to propionate and acetate. These data suggest that fermentation of lactate directly to acetate (an alternative lactate fermentation pathway) was not significant in the enrichment culture used in this study. Accumulation of acetate was not observed in uninhibited culture series and the carbon flow ended with methane production. Lactate fermentation and production of propionate and acetate at a molar ratio of ca 2:1 and further conversion of both propionate and acetate to methane was recently reported for a lactate-fed, trichlorobenzene-dechlorinating methanogenic consortium (Middeldorp *et al.*, 1997).

At the relatively high levels of electron donor added to the enrichment culture as compared to the HCBD concentration, the HCBD biotransformation kinetics were zero-order with respect to the available reducing equivalents. Consequently, it was not apparent that the addition of lactate and methanol was any more favorable with respect to HCBD dechlorination than the addition of methanol alone. Previous research has shown that microbial reductive dechlorination of chloroalkenes requires an active primary metabolic process to supply the necessary reducing equivalents (Pavlostathis and Zhuang, 1991, 1993; Zhuang and Pavlostathis, 1995). However, data from the methanol only series in the assay, which compared methanol and methanol plus lactate as electron donors, suggest that HCBD biotransformation was not correlated to the cultures' methanogenic activity (Fig. 4). Even after methane production stopped at 2.4 days of incubation in the methanol only series, HCBD biotransformation continued at approximately the same rate as in the methanol plus lactate-amended series where electron donor utilization and gas production continued beyond 2.4 days. It is possible that an endogenous source of electrons, such as microbial death and decay, provided sufficient reducing equivalents to sustain the HCBD biotransformation rate beyond the incubation time corresponding to the depletion of the exogenous electron donor (methanol). Wrenn and Rittmann (1995) noted that reductive dehalogenation frequently occurs in the absence of exogenous electron donors, and included biomass decay as a source of endogenous electrons in their proposed model of substrate effects on the kinetics of dehalogenation. Current research has focused on the importance of molecular hydrogen (H_2) as the ultimate source of reducing equivalents for reductive dechlorination processes (Smatlak *et al.*, 1996; Ballapragada *et al.*, 1997; Fennell and Gossett, 1998). The fermentation of lactate and propionate, as well as methanogenesis from methanol and acetate, have been characterized as hydrogenogenic reactions (Lovley and Ferry, 1985; Cord-Ruwisch and Ollivier, 1986; Zehnder and Stumm, 1988; Thiele and Zeikus, 1988; DiStefano *et al.*, 1992; Brock *et al.*, 1994), and thus are potential sources of molecular hydrogen for dechlorination.

The observed higher HCBD dechlorination rate at the 1.5 mg HCBD/l level (as compared to that at the 0.4 mg HCBD/l level) is noteworthy in light of the inherent toxicity of HCBD. The observed rate enhancement may, in fact, be related to selective or partial inhibitory effects of HCBD to the enrichment consortium. Recent work has shown that the values of the H_2 half-velocity constants of dechlorinating organisms are as much as an order of magnitude lower than those for methanogens. As a result, dechlorination rates can be enhanced by maintaining low H_2 levels via a slow production rate, thus minimizing competition for hydrogen from methanogens (Smatlack *et al.*, 1996; Ballapragada *et al.*, 1997; Fennell and Gossett, 1998). The rate of propionate fermentation—and therefore the concomitant production of H_2 based on theoretical stoichiometry—decreased with increasing levels of HCBD (Fig. 5). Competition for H_2 was also likely diminished by direct inhibition of methanogenesis at the relatively high levels of HCBD. The coexistence of these two conditions (i.e., slow H_2 production and lower competition for H_2 due to methanogenic inhibition) at relatively high levels of HCBD may have led to higher HCBD dechlorination rates. In a previous study, the inhibition of methanogenesis by high levels of PCE was associated with a shift in electron flow towards dechlorination resulting in enhanced PCE biotransformation in a mixed culture (DiStefano *et al.*, 1991).

Addition of BES to the enrichment culture resulted in inhibition of methanogenesis. HCBD dechlorination, however, was unaffected. BES is a structural analog and competitive inhibitor of the enzyme methyl-coenzyme M reductase found only in methanogens and which catalyzes the final step of methane formation (Sparling and Daniels, 1987; DiMarco *et al.*, 1990). Since this enzyme is present in all methanogens, regardless of the carbon source, the use of BES in this study was to block methane formation from either H_2/CO_2, acetate, or methanol. Several investigators have reported BES inhibition of microbial reductive dechlorination in methanogenic cultures leading to the conclusion

that methanogens were directly or indirectly involved in the observed dechlorination reactions (Fathepure and Boyd, 1988; Freedman and Gossett, 1989; DiStefano *et al*., 1992). A recent study showed inhibition of non-methanogenic, chloroethene-dechlorinating bacteria by BES (Löffler *et al*., 1997). However, inhibition of chloroethene dechlorination was not found in all of the non-methanogenic cultures tested by these investigators. Therefore, simultaneous BES inhibition of both methanogenesis and dechlorination in mixed cultures does not necessarily constitute proof that methanogens are involved in the dechlorination reactions. In the present study, however, even in the case of complete inhibition of methanogenesis by BES, the rate of HCBD dechlorination was not affected, indicating that methanogens were probably not involved in HCBD dechlorination.

In conclusion, the methanogenic enrichment culture developed in this study mediated the sequential reductive dechlorination of HCBD to predominantly trichloro- and dichloro-1,3-butadiene isomers, as well as traces of a monochloro-1,3-butadiene congener. Enhanced HCBD dechlorination rates were observed under higher initial HCBD levels (0.4 versus 1.5 mg HCBD/l). Methanogens did not appear to be responsible for the observed HCBD dechlorination in the enrichment culture. Although at least partial dechlorination of HCBD took place under the anaerobic conditions of the mixed, enrichment culture, the detoxification efficiency of this process remains unclear because the potential inhibitory effects of the HCBD transformation products are unknown. Further testing of the potential inhibitory effect of the HCBD transformation products as well as characterization of the enrichment culture may lead to insights critical to the optimization of conditions for the bioremediation of HCBD-contaminated soil, water, and sediments.

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